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# Phase 1 and 2 Metabolism in Freshly Isolated Hepatocytes and Subcellular Fractions from Rat, Mouse, Chicken and Ox Livers†

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**Abstract:** In toxicological studies hepatocytes offer an excellent alternative to whole-animal experiments, provided their metabolic competence has been established. We have compared Phase 1 and 2 metabolism in rat, mouse, chicken and ox liver microsomes and cytosol with freshly isolated hepatocytes. The relative amounts of total cytochrome P450 in microsomes and hepatocytes were equivalent. Rat liver had the highest P450 content while chicken liver had the lowest content (148·2(±75·7) and 20·6(±11·5) pmol mg$^{-1}$ hepatocellular protein, respectively). The metabolism of testosterone was assessed to determine selective cytochrome P450 isoenzyme activities. Only two metabolite products were common to all four species, namely 6$\beta$-hydroxytestosterone (6$\beta$-OHT) and androstenedione (ASD), which co-eluted with 6-dehydrotestosterone (6DHT). 16$\alpha$-OHT was present in all incubations except for ox microsomes. The rate of metabolism of testosterone was generally lower in microsomes than hepatocytes, with the exception of the ox, but the pattern and quantity of metabolite formation was similar. The quantity of total products formed was 15- to 27-fold higher in rat and mouse livers than in chicken or ox. The major product formed in freshly isolated hepatocytes from mice and chickens was ASD/6DHT which accounted for 60% and 76% of the total metabolites, respectively. ASD/6DHT formation accounted for only 33% and 17% of the total metabolites formed by rat and ox hepatocytes, respectively. 2$\alpha$-OHT production occurred in rat and mouse hepatocytes (14% of the total metabolites in rat and 7% in mouse hepatocytes) but was lacking in chicken or ox cells. The stability of P450 isoforms in culture was species-dependent. Rat and mouse hepatocyte cultures lost 54% and 31% of their initial P450 content after 72 h, while there was no loss in chicken hepatocytes over the same period.

There was a good correlation between the relative glutathione *S*-transferase (GST) activities in cytosol and freshly isolated hepatocytes. Mouse liver exhibited highest GST activity (664·2(±203·5)) compared with rat, chicken or ox (320·4(±64·0), 341·5(±13·9) and 256·3(±109·9) nmol min$^{-1}$ mg$^{-1}$ cytosolic protein, respectively).

Key words: species differences, hepatocytes, cytochrome P450, glutathione *S*-transferase, *in vitro* toxicology

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## 1 INTRODUCTION

Suspensions and cultures of hepatocytes are now well established in pharmacology and toxicology as alternatives to the use of whole-animal experiments.[1,2] Fewer animals are used in in-vitro studies and the conditions in which the compounds are tested can be precisely controlled. Although the majority of metabolic and toxicological data has been obtained in the rat, the suitability of this species is questionable when extrapolating data to other species. Hepatocytes isolated from food-producing animals (ruminants, fowl and fish) would be of great value in short-term predictive assays to establish routes of metabolism and mechanisms of toxicity of veterinary, pharmaceutical and agrochemical compounds. The potential to form drug residues may also be determined using hepatocytes, an important issue to consider at the start of the development of a veterinary drug.

It has long been known that there are differences in metabolism between species.[3,4] However, there are relatively few reports of toxicological and metabolic studies carried out in farm animals and even fewer using isolated hepatocytes. We favour the use of hepatocytes rather than subcellular fractions for a number of reasons: they are intact viable cells only hours removed from the in-vivo situation and as such contain enzyme systems and associated cofactors so that they may carry out sequential Phase 1 and 2 metabolic reactions. Hepatocytes contain physiological concentrations of cofactors, unlike subcellular fractions, which require the provision of exogenous cofactor-generating systems.[5] Hepatocytes are essentially nonproliferating cells,[6] exhibiting the many differentiated functions seen *in vivo* and can be cultured for days. Thus, hepatocytes may be used to determine the mechanisms of action of cytotoxic compounds, enzyme inducers, genotoxic compounds and cell proliferators.

We have measured Phase 1 and 2 metabolism in freshly isolated hepatocytes, microsomes and cytosol from the livers of rats, mice, chickens and ox. Selective cytochrome P450 isoenzyme activity was determined using metabolism of testosterone and glutathione *S*-transferase activity was measured using the broad-spectrum substrate, 1-chloro-2,4-dinitrobenzene.

## 2 METHODS

### 2.1 Materials

Leibovitz Glutamax I medium and Hank's balanced salt solution (HBSS) were obtained from Gibco BRL, Paisley, Scotland, collagenase A (0·22 U $mg^{-1}$) and B (0·75 U $mg^{-1}$) from Boehringer Mannheim Corp. Ltd, Worthing, Sussex. Folin and Ciocalteu's phenol reagent and Triton X-100 were from Merck, Poole, Dorset, UK. Digitonin was a gift from Dr E. Eliasson. All other chemicals used were from either Sigma Chemical Co. Ltd, Poole, Dorset or Aldrich Chemical Co. Ltd, Gillingham, Dorset and were of the highest grade obtainable.

### 2.2 Animals

Male Fischer 344 rats (150–250 g) and male CD-1 mice (20–30 g), were purchased from Charles River UK Ltd, Manston, Kent and were fed on Labsure CRM rat pellets from Special Diet Services, Witham, Essex. Male SPF Torbay chickens (6–8 weeks, 450–600 g) were from Wickham Laboratories Ltd, Southampton, Hants. Ox (550–650 kg) livers were transported from a registered abattoir in phosphate-buffered saline at 4°C and used within 4 h of the death of the animal.

### 2.3 Cell isolation and culture

Rat, mouse and chicken hepatocytes were isolated by a two-step collagenase A perfusion technique.[7] Ox hepatocytes were isolated according to Van't Klooster *et al.*[8] using collagenase B. Initial cell viability and number of hepatocytes were assessed by Trypan blue (TB) exclusion. The initial viabilities of rat, mouse, chicken and ox hepatocytes were 96·6($\pm$2·6), 93·2($\pm$1·7), 92·1($\pm$4·4) and 82·5($\pm$13·2)%, respectively. Cells were diluted to the required density (see Section 3) and plated in 35-mm Falcon plastic culture dishes in 1 ml Leibovitz (L15) Glutamax I medium supplemented as described previously.[9] Cells were maintained in a humidified atmosphere at 37°C and 5% carbon dioxide. The medium was replaced with fresh complete L15 4 h after plating and then at 24-h intervals where necessary. Cell cultures received no additional treatment. Attachment of hepatocytes to plastic culture dishes,[9] total P450 content and testosterone metabolism were assessed at 0, 24, 48 and 72 h after plating.

### 2.4 Microsome preparation

Livers were chopped and homogenised in 15 mM Tris buffer containing 0·25 M sucrose and 0·1 mM EDTA, pH 6·8 (3 ml $g^{-1}$ liver weight). The homogenate was centrifuged (10 000*g*) for 16 min at 4°C and the supernatant was kept. The pellet was resuspended in homogenisation buffer and centrifuged (10 000*g* for 16 min at 4°C). The pellet was discarded, the supernatant was amalgamated with the first supernatant obtained and then centrifuged (100 000*g*) for 90 min at 4°C. The supernatant (cytosol) was removed and snap frozen in liquid nitrogen. The pellet (microsomes) was resuspended in 1 volume of 50 mM potassium dihydrogen phosphate buffer containing 0·1 mM EDTA and

200 ml $litre^{-1}$ glycerol, pH 7·4 and snap frozen. Protein content was determined by the method of Lowry *et al.*[10]

## 2.5 Attachment and viability assay

The number of cells attached at each time point was determined by measuring the LDH activity[11] in attached cells and expressing this as a percentage of the total LDH activity in the cells originally plated.[9] The number of cells attached was calculated by multiplying the total number of cells plated by the percentage attachment at that time point. These values were used to express total P450 and hydroxytestosterone production per $10^6$ cells. After 72 h in culture, the attachment of rat, mouse and chicken hepatocytes was 85·9(±6·3), 74·2(±4·0) and 70·1(±3·9)%, respectively (mean ± SD, $n = 3$). Culture efficiency of ox hepatocytes was not determined.

## 2.6 Total cytochrome P450

The total P450 content of hepatocytes[12] and microsomes[13] was measured as described previously. The extinction coefficient for cytochrome P450 was taken to be 91 $mM^{-1}$ $cm^{-1}$.[13] P450 content was expressed as pmol $mg^{-1}$ protein or pmol per $10^6$ cells.

## 2.7 Testosterone metabolism in whole cells and microsomal incubations

Hepatocyte suspensions and cultures were incubated at 37°C with HBSS (1 ml) containing 0·25 mM testosterone and the 5α-reductase inhibitor, 17α-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (4-MA, 1 μM). Microsomes were diluted to 1 mg protein $ml^{-1}$ in HBSS containing 1 μM 4-MA. The NADPH generator system added was 2·5 units $ml^{-1}$ glucose-6-phosphate dehydrogenase, 5 mM $NADP^+$ and 50 mM glucose-6-phosphate. Rat and mouse hepatocytes and microsomes were incubated for 15 min and chicken and ox for 30 min. Metabolism was terminated by the addition of dichloromethane (6 ml) to cell suspensions or by transfer of the HBSS from culture plates to dichloromethane. Culture dishes were placed on ice for 5 min, the cells harvested and added to the corresponding HBSS/dichloromethane mixture. 11α-Hydroxyprogesterone (2·5 μg per sample) was added as an internal standard to the samples (with which to compare the peak area of the metabolites), which were then mixed and centrifuged at 2000 rev $min^{-1}$ for 5 min. The aqueous phase and cellular proteins were aspirated and discarded and the remaining dichloromethane fractions were evaporated to dryness under a stream of nitrogen. Residues were reconstituted in methanol + water (1 + 1 by volume) and analysed by HPLC.[14] Metabolites were separated on a reverse phase Spherisorb S5ODS2-250A column (25 cm × 4·6 mm ID) with a 10-mm C18 guard column. The mobile phases consisted of A: methanol + water + acetonitrile (39 + 60 + 1 by volume) and B: methanol + water + acetonitrile (80 + 18 + 2 by volume). The gradient elution system was as follows: 0 min B = 30%, 15 min B = 30%, 22 min B = 35%, 27 min B = 50%, 30 min B = 90%, 35 min B = 90%, 40 min B = 30%, 50 min B = 30%. Metabolites were detected by UV at 254 nm. Retention times of testosterone metabolites were: 7α-OHT = 10 min; 6β-OHT = 11·6 min; 16α-OHT = 13 min; 16β-OHT = 17·3 min; 2α-OHT = 19·6 min; 11α-hydroxyprogesterone = 24 min; ASD and 6DHT = 30 min; testosterone = 31·5 min. Each metabolite peak area was compared with that of the internal standard, giving peak area ratio (PAR) values. Testosterone metabolite formation was expressed as the peak area ratio (PAR) × 1000 per min per $10^6$ cells or PAR $min^{-1}$ $nmol^{-1}$ P450.

## 2.8 Glutathione *S*-transferase assay

Glutathione *S*-transferase activity was measured according to Habig *et al.*[15] Rat and ox hepatocytes were diluted to $10^6$ cells $ml^{-1}$, chicken cells to 3 × $10^6$ cells $ml^{-1}$ and mouse cells to 0·3 × $10^6$ cells $ml^{-1}$ in HBSS containing 1 mM glutathione. Cytosol was diluted to 1 mg protein $ml^{-1}$ in HBSS containing 1 mM glutathione. The reaction was initiated by the addition of 1-chloro-2,4,dinitrobenzene (50 μM final concentration) and the initial rate of glutathione conjugation was measured with a Shimadzu MPS 2000 spectrophotometer set at 340 nm.

# 3 RESULTS AND DISCUSSION

## 3.1 Culture of hepatocytes

An important factor in the culture of hepatocytes is the density at which the cells are plated on to culture plates. If too many cells are cultured, the excess die and release lytic components into the culture plate. Table 1 compares the protein contents and cellular volumes of hepatocytes from different species. Rat and ox hepatocytes have a similar volume and protein content and were plated at $10^6$ cells per plate. Mouse hepatocytes were 3·2-fold larger in volume (but not protein content) than rat so that only 0·3 × $10^6$ cells were plated to cover the same area. In contrast, chicken hepatocytes are much smaller than rat hepatocytes and therefore 3 × $10^6$ cells were required to achieve confluency.

**TABLE 1**
Cellular Protein Content and Optimum Plating Densities of Hepatocytes from Different Species

| *Species* | *Cell protein content*[a] *(mg/10⁶ cells) (±SD)* | *Cell volume as a ratio of rat cell volume*[b] | *Optimum plating density No. cells (×10⁶ cells)/35-mm plate* |
|---|---|---|---|
| F344 rat | 1·14 (±0·34) ($n$ = 15) | 1 ($n$ = 40) | 1 |
| CD1 mouse | 1·74 (±0·46) ($n$ = 10) | 3·2 ($n$ = 33) | 0·3 |
| Chicken | 0·31 (±0·14) ($n$ = 12) | 0·2 ($n$ = 36) | 3 |
| Ox | 0·70 (±0·13) ($n$ = 9) | 0·9 ($n$ = 36) | 1 |

[a] $n$ = number of animals used.
[b] $n$ = number of cells measured. The relative volume of hepatocytes was calculated by measuring the diameter of cells from different species photographed at the same magnification.

### 3.2 Phase 1 metabolism

*3.2.1 Total cytochrome P450*
Figure 1 compares the total P450 content of microsomes derived from the livers of rats, mice, chickens and ox and freshly isolated hepatocytes. The overall pattern is similar in microsomes and cells, with rat liver having the largest amount of P450 and chicken the lowest P450 content. The pattern of P450 contents expressed per $10^6$ cells in different species was different from P450 content expressed per mg protein. Mouse hepatocytes had a lower P450 content than rat hepatocytes when values were expressed per mg protein but a higher content than rat hepatocytes when expressed per $10^6$ cells. We attribute this difference to the larger cellular volume of mouse hepatocytes which thus contain more endoplasmic reticulum than rat hepatocytes. The terms used for the expression of data are very important, as protein contents may vary in cell cultures, especially when treated with enzyme inducers. Values expressed per $10^6$ cells may be more applicable in these situations.

*3.2.2 P450 isoenzyme activities*
Using metabolism of testosterone as an indicator of specific isoenzyme activities, qualitative and quantitative differences in metabolism between species were measured in hepatocytes and compared with microsomes. Table 2 shows that only two metabolic products of testosterone were common to hepatocytes and microsomes of all four species tested, namely 6β-hydroxytestosterone (6β-OHT), formed by CYP 3A in the rat,[14] and the two co-eluting metabolites, androstenedione (ASD) and 6-dehydrotestosterone (6DHT), formed by CYP 2B1/2 (phenobarbital-induced only) and CYP 2C in the rat.[14,16] 16α-OHT was formed in hepatocytes and microsomes of rats, mice and chickens; however, this metabolite could not be detected in ox microsomes, despite its formation in corresponding hepatocyte incubations. 16β-OHT (indicative of CYP 2B metabolism in the phenobarbital-induced rat) was notably absent from rat and mouse livers but present in both chicken (4% of total metabolite production) and ox livers (20% of total metabolite production) and 2α-OHT (CYP 2C11 in male rat liver) was present in rat and mouse but not in chicken or ox liver. There were three unknown metabolites, one (unknown 1, retention time 15·1 min) produced only in rat and mouse, a second only in chicken and ox (unknown 3, retention time 20·2 min) and a third (unknown 2, retention time

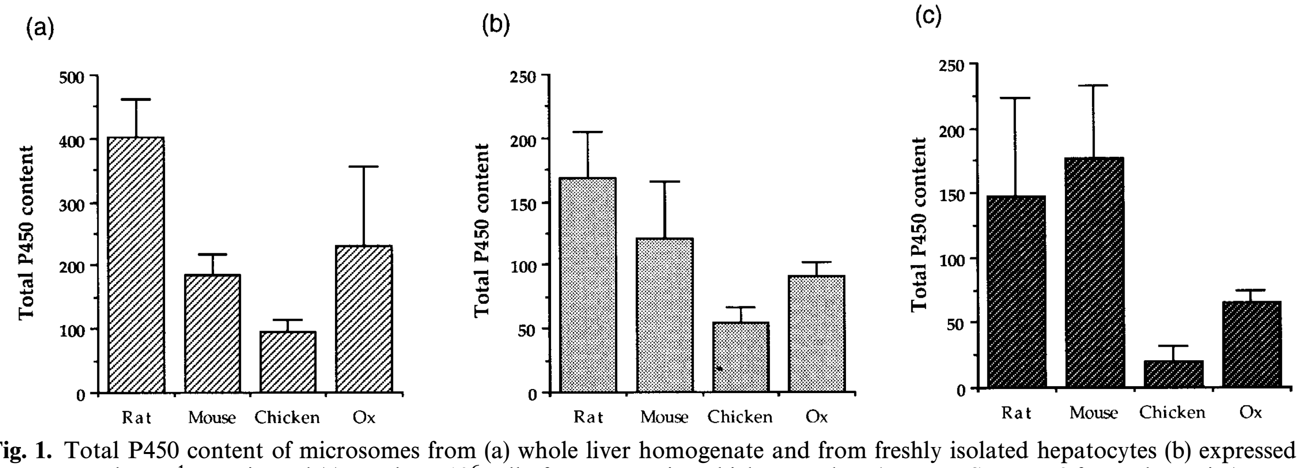


**Fig. 1.** Total P450 content of microsomes from (a) whole liver homogenate and from freshly isolated hepatocytes (b) expressed as pmol $mg^{-1}$ protein and (c) pmol per $10^6$ cells, from rats, mice, chickens and ox (mean ± SD, $n$ = 3 for each species).

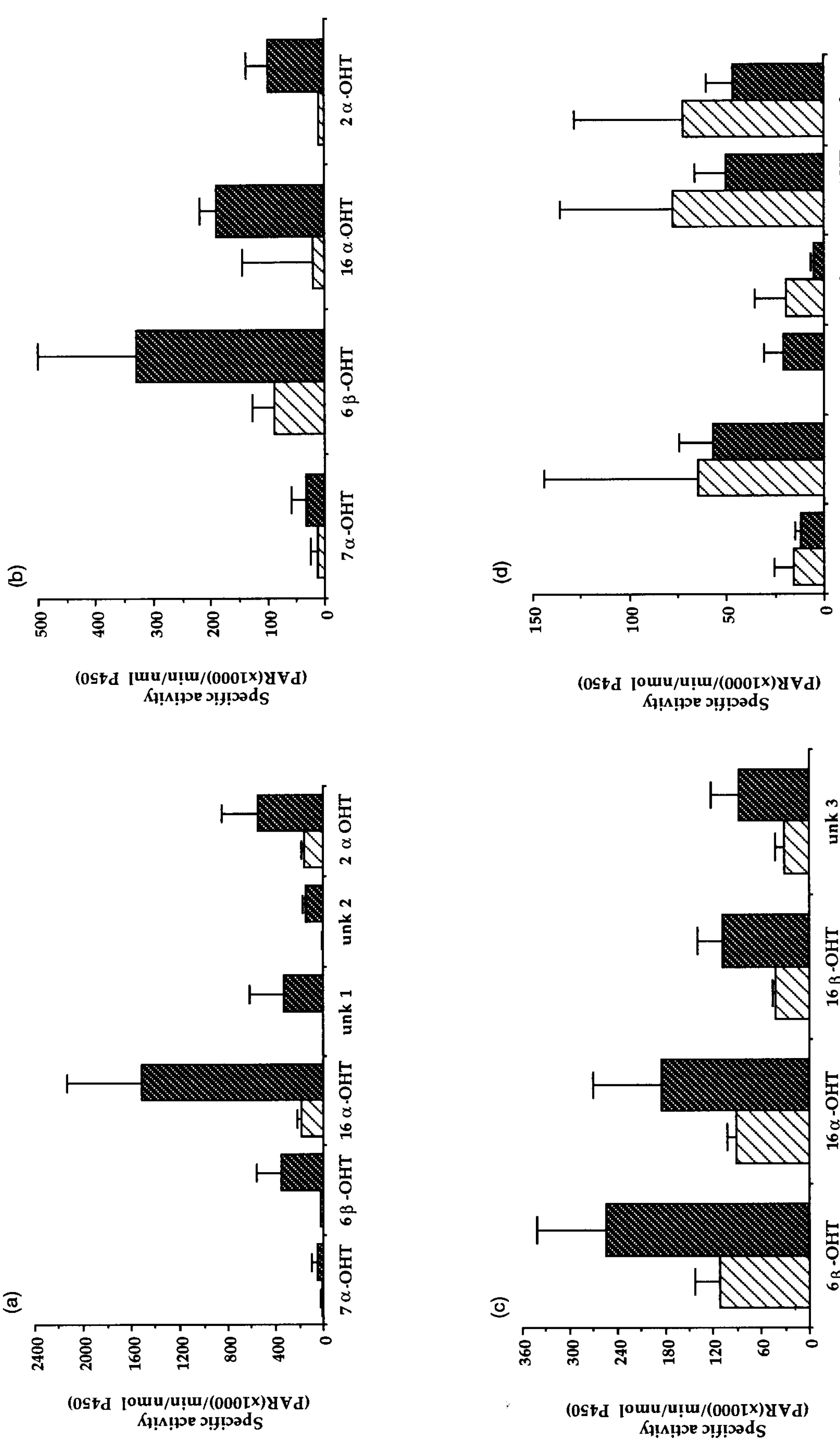


**Fig. 2.** Comparison of testosterone metabolism in liver (▨ microsomes and (▩) freshly isolated hepatocytes of (a) rat, (b) mouse, (c) chicken and (d) ox (mean ± SD, $n = 3$ for each species).

**TABLE 2**
Testosterone Metabolite Profiles in Liver Microsomes and Hepatocytes from Different Species

| | *Metabolite*[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Species* | *7α-OHT*[b] *(10·0)*[c] | *6β-OHT (11·6)* | *16α-OHT (13·0)* | *Unk 1 (15·1)* | *Unk 2 (16·4)* | *16β-OHT (17·3)* | *2α-OHT (19·6)* | *Unk 3 (20·2)* | *ASD/6DHT (30·0)* |
| F344 rat | + | + | + | + | − | − | + | − | + |
| CD1 mouse | + | + | + | + | − | − | + | − | + |
| Chicken | − | + | + | − | − | + | − | + | + |
| Ox | + | + | ±/− | − | + | + | − | + | + |

[a] + = present in microsomes and hepatocytes. − = absent in microsomes and hepatocytes. ±/− = present in hepatocytes but absent in microsomes.
[b] Unk = unknown metabolite, ASD = androstenedione, 6DHT = 6-dehydrotestosterone, OHT = hydroxytestosterone.
[c] Numbers in parentheses indicate HPLC retention time in minutes.

16·4 min) which was unique to ox liver.

Figure 2 shows the specific activity of testosterone hydroxylases (excluding ASD and 6DHT) per nmol cytochrome P450 in microsomes and freshly isolated hepatocytes. The metabolic profiles of microsomes and hepatocytes were similar for all species. The activities of P450 were higher in rat, mouse and chicken hepatocytes than in the corresponding microsomes (Fig. 2(a), (b) and (c), respectively). The specific activities in ox microsomes were not significantly different from those in freshly isolated hepatocytes, although 16α-OHT production occurred in hepatocytes but not in microsomes (Fig. 2(d)). ASD and 6DHT production was significantly lower in microsomes than in hepatocytes (1585·9(±476·9), 1363·4(±383·4), 2024·6(±564·4) and 42·5 PAR(×1000) $min^{-1}$ $nmol^{-1}$ in rat, mouse, chicken and ox hepatocytes, respectively, compared to 55·3(±9·4), 49·3(±10·7), 267·9(±7·1) and 30·0(±9·9) PAR(×1000) $min^{-1}$ $nmol^{-1}$ in rat, mouse, chicken and ox microsomes, respectively), indicating the involvement of cytosolic enzyme(s) in the production of these metabolites.

The total formation of testosterone metabolites (Table 3) was highest in rat and mouse hepatocytes and was 15- to 27-fold higher than in chicken or ox hepatocytes. The major peak formed in mouse and chicken liver was ASD/6DHT accounting for 60% and 76% of the total metabolite formation, respectively. There were two major products formed in rat hepatocytes, namely, ASD/6DHT and 16α-OHT, accounting for 33% and 32% of the total metabolites formed, respectively. In contrast, metabolism in ox hepatocytes was more evenly spread, 6β-OHT, 16α-OHT, 16β-OHT, unknown 3 and ASD/6DHT accounting for 22, 16, 20, 18 and 17% of the total products formed, respectively. 16α-OHT and 2α-OHT production was higher in rat hepatocytes (yielding 46% of total metabolites) than in mouse, chicken and ox cells (yielding only 7–20% of total metabolites) but production of 6β-OHT was equivalent in all four species (giving 7–22% of the total metabolism). The stability of CYP isoenzymes in culture was species-dependent (Table 4). Chicken hepatocytes, despite their low P450 content, lost no activity over 72 h in culture, while rat and mouse hepatocytes lost 54% and 31% of their initial activity over the same period.

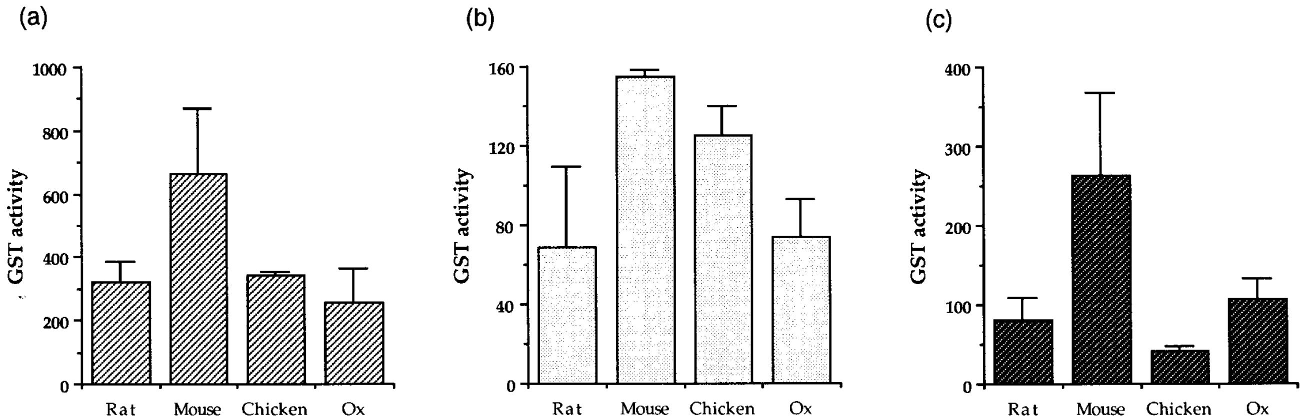


**Fig. 3.** Glutathione *S*-transferase activity in cytosol (a) from whole liver homogenate and from freshly isolated hepatocytes (b) expressed as nmol product formed $min^{-1}$ $mg^{-1}$ protein and (c) nmol product formed per min per $10^6$ cells from rats, mice, chickens and ox (mean ± SD, $n = 3$ for each species).

**TABLE 3**
Quantities of Testosterone Metabolites Formed (on a Cell Basis) in Freshly Isolated Hepatocytes

| | *Peak area ratio ( × 1000) per $10^6$ cells per min (±SD) [% total metabolism]* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *16α-OHT*[a] | *2α-OHT* | *6β-OHT* | *16β-OHT* | *Unknown 3* | *ASD/6DHT* | *Total metabolites*[b] |
| F344 rat ($n = 4$) | 111·0 (±23·1) [32] | 49·4 (±1·1) [14] | 22·8 (±15·9) [7] | 0·0 (±0·0) [0] | 0·0 (±0·0) [0] | 115·7 (±33·1) [33] | 347·3 (±69·8) |
| CD1 mouse ($n = 4$) | 42·1 (±12·7) [13] | 23·6 (±5·3) [7] | 56·9 (±20·4) [18] | 0·0 (±0·0) [0] | 0·0 (±0·0) [0] | 189·7 (±22·2) [60] | 317·5 (±122·6) |
| Chicken ($n = 3$) | 1·4 (±0·3) [7] | 0·0 (±0·0) [0] | 2·0 (±0·2) [10] | 0·9 (±0·3) [4] | 0·7 (±0·1) [3] | 15·9 (±0·7) [76] | 20·9 (±0·6) |
| Ox ($n = 5$) | 2·0 (±2·0) [16] | 0·0 (±0·0) [0] | 2·8 (±1·2) [22] | 2·5 (±1·1) [20] | 2·3 (±0·9) [18] | 2·1 (±1·2) [17] | 12·8 (±4·5) |

[a] For abbreviations, see Table 2.
[b] Total metabolites = sum of PAR × 1000 per $10^6$ cells per min for all metabolites formed.

**TABLE 4**
Stability of Testosterone 6$\beta$-Hydroxylation in Rat, Mouse and Chicken Hepatocytes

| *Time in culture (h)* | *Percentage of initial 6β-hydroxylase activity (±SD)*[a] | | |
|---|---|---|---|
| | *F344 rat* | *CD1 mouse* | *Chicken* |
| 0 | 100 [347·0 (±210·9)][b] | 100 [416·4 (±252·9)] | 100 [253·3 (±88·9)] |
| 24 | 81 (±10) | 101 (±12) | 103 (±2) |
| 48 | 71 (±51) | 84 (±28) | 100 (±2) |
| 72 | 46 (±34) | 69 (±20) | 109 (±16) |

[a] $n = 3$ for each species.
[b] Values in brackets are initial 6$\beta$-OHT activities expressed as PAR($\times$1000) $min^{-1}$ $nmol^{-1}$ P450.

### 3.3 Phase 2 metabolism

Glutathione *S*-transferase (GST) activity was measured in cytosol from whole liver homogenate and compared with that in freshly isolated hepatocytes (Fig. 3). The pattern of metabolism was similar in hepatocytes and cytosol, with the mouse liver exhibiting the highest rate of conjugation. The activity of mouse GST relative to that in rat, chicken and ox hepatocytes increased significantly when these values were expressed per $10^6$ cells. Again, we have attributed this difference to the larger cellular volume of mouse cells.

## 4 CONCLUSIONS

These results suggest that metabolic studies must be carried out on a species-to-species basis, since prediction of compound metabolism from rat or mouse may be questionable. The relative rates of 1-chloro-2,4-dinitrobenzene conjugation with glutathione by cytosolic transferases from different species were similar to those in hepatocyte suspensions. Metabolism of testosterone in rat and mouse hepatocytes was 15- to 27-fold higher than in chicken and ox hepatocytes, a finding supported by others,[17] and the cytochrome P450s involved varied markedly between species. The metabolism of testosterone was qualitatively similar in hepatocytes and subcellular fractions with all species used, since metabolites produced in microsomes from each species were also produced in their corresponding hepatocytes. Hepatocytes may be a more appropriate tool for studying metabolism in ox livers, however, since 16$\alpha$-OHT was detected only in hepatocytes and not in microsomes obtained from the same liver. The metabolism of testosterone was higher in hepatocytes than in microsomes, a finding also reported by others,[1] emphasising the suitability of hepatocytes for use in metabolic assays.

## ACKNOWLEDGEMENT

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